Lipid from electronic cigarette smoke both with and without nicotine induced pro-inflammatory macrophage polarization and disrupted phagocytosis

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Abstract

Clinical cases and experimental evidence show that electronic cigarette (ECIG) induce serious adverse health effects but underlying mechanisms much remain to be uncovered. Based on recent exploratory evidence, investigating the effects of ECIG on macrophages can broadly define potential mechanisms by focusing on the effect of ECIG exposure with or without nicotine. Here we investigated the effect of ECIG-smoke exposure on macrophages (MQ) phenotype, inflammatory response, and function of macrophages.

MQ were cultured at air liquid interface and exposed to ECIG smoke. Oxidative stress was determined by reactive oxygen species (ROS), heat shock protein 60 (HSP60), glutathione peroxidase (GPx) and heme oxygenase1 (HMOX1). Lipid accumulation was ensured by lipid staining and lipid peroxidation was measured by level of malondialdehyde (MDA). MQ polarization was identified by surface expression markers CD86, CD11C and CD206 as well as pro-inflammatory and anti-inflammatory cytokines in gene and protein level. Phagocytosis of E. coli by MQ were investigated by fluorescence-based phagocytosis assay.

ECIG smoke exposure in presence or absence of nicotine induced oxidative stress as ROS, HSP60, GPx, GPx4 and HMOX1 was upregulated in MQ. ECIG exposure induced accumulation lipids and the lipid peroxidation product MDA in MQ. Pro-inflammatory MQ (M1) markers CD86 and CD11C but not anti-inflammatory MQ (M2) marker CD206 were upregulated in response to ECIG exposure. In addition, ECIG induced pro-inflammatory cytokines IL-1beta and IL-8 in gene level and IL-6, IL-8, and IL-1beta in protein level whereas ECIG exposure downregulated anti-inflammatory cytokine IL-10 in protein level. Phagocytosis activity of MQ was downregulated by ECIG exposure. shRNA mediated lipid scavenger receptor CD36 silencing inhibited ECIG-induced pro-inflammatory MQ polarization and recovered phagocytic activity of MQ.

ECIG exposure alter lung lipid homeostasis and thus induced inflammation by inducing M1 type MQ and impair phagocytic function, which could be a potential cause of ECIG-induced lung inflammation in healthy and inflammatory exacerbation in disease condition.

Introduction

The trends of using electronic cigarettes (ECIG) are increasing. First generation of ECIG was introduced in 2003 and became available commercially in 2007 in the US and spread through the countries(1). The use of ECIG is dramatically increasing and it is predicted that it will supersede the traditional smoking in next ten years(2). Although ECIG was expected as a safer alternative than the traditional smoking, in US by the mid of February 2020, a total 2807 ECIG users got severe lung injury, hospitalized and 68 deaths were confirmed(3, 4). The emergence of a case report from 98 patients related to lung illness reported various clinical symptoms and toxicity(3). The patients reported respiratory symptoms, cough, chest pain, gastrointestinal symptoms, and constitutional symptoms. Clinically, 100% of patients with vaping
associated lung injury experience constitutional symptoms such as fever, chills, and weight loss, whereas respiratory symptoms were present in 97% of patients(4). In addition, bilateral infiltrates on chest X-ray were reported. Mild and non-specific inflammation as well as foamy or lipid laden macrophages were identified(4–7). Moreover, mice studies identified that chronic ECIG smoke exposure downregulated macrophage mediated innate immunity (7). Most importantly, the study showed lipid dysregulation in response to ECIG smoke exposure. Emerging data on human and mouse, indicated an important role of macrophages in ECIG-induced toxicity. ECIG-induced toxicity and inflammatory responses were nicotine independent (7, 8) whereas nicotine dependent TLR2 reduced level was reported(9).

Use of ECIG might cause an unknown vaping related lung disease as well as potential cause of cardiovascular diseases after chronic exposure(10, 11). First comprehensive study in human volunteers that examined acute vascular impact against ECIG has been reported(11). The study identified that ECIG induced arterial stiffness, which is an early indicator of cardiovascular disease (CVD) (11). According to experimental studies, ECIG induce inflammatory responses, oxidative stress, cell death and DNA damage as well as reduced phagocytosis of bacteria have been reported(8, 12). However, in the proximal airways and alveoli, macrophages are the specialized resident immune cells that encounter and clean up inhaled particles like pollutants or antigens and thus regulate cellular homeostasis(9). Alteration in function of macrophages in airways can lead to the pathogenesis of lung disease such as COPD and increase susceptibility to infection such as pneumonia(9, 13).

ECIG-induced toxicity and immune responses including antimicrobial activity showed to be nicotine independent in and experimental study(7). Toxicity of ECIG is now established, but not the mechanisms behind disease development. In ECIG smoke, around 5,000 different chemicals were identified, and recent exploratory evidence investigating the effects of ECIG on alveolar macrophages could not broadly define potential mechanisms by focusing on nicotine dependent and independent effects, and disruption of lipid metabolism by the altered lipid processing(9).

Although the recommendations of the European Respiratory Society task force on ECIG research included the need for identification of molecular patterns as well as studies characterizing the health effects and toxicity of ECIG flavorings(5), but still underlying mechanism of ECIG-induced adverse health effect much remain to be uncovered. However, we found in an in vitro study that ECIG with or without nicotine induced pro-inflammatory effects and DNA methylation(14). Furthermore, we recently identified significantly higher prevalence of cough and higher mucus production among ECIG users in comparison to non-ECIG users(15). However, information on the effect of ECIG against the immune system is still lacking (51). Furthermore, comparing the effects of ECIG exposure of macrophages in presence or absence of nicotine with same flavor is missing. Despite commonality of lipid accumulation in lung in ECIG users, the physiological importance of accumulation of lipid and whether such phenomenon can be recapitulated in experimental systems remain to be elucidated. Here we investigated macrophage polarization, inflammatory effects and impaired functions of macrophages and lipid homeostasis in connection with ECIG-exposure (16).
Methods And Materials

Cell culture

Buffy coat was collected from Karolinska University hospital, Sweden, and monocytes were isolated by negative selection kit (Stem cell technologies, UK). Isolated monocytes were stimulated with 50ng/ml of GM-CSF (Immunotools, Germany) for 5 days to differentiate into macrophages. Macrophage differentiation was confirmed by light microscopic visual observation and CD11B staining. The differentiated macrophages were trypsinized and seeded in 12 wells transwell inserts with 0.4µm pore size (Corning, Sigma Aldrich, Sweden). After overnight incubation, cell culture media was removed from the apical side to start culturing at air-liquid interface (ALI) and basal side was filled with the RPMI complete media with 10% FBS and 1% penicillin/Streptomycin.

ECIG exposure

Macrophages were exposed to ECIG smoke according to the earlier established protocol (14). According to the earlier study, we selected the ECIG flavor 2 (ripe strawberry, sweet apples and tart kiwi) which was more toxic than the flavor 1 (14). Shortly, macrophages in 12 well plates were placed in a glass jar with 3L desiccator volume, maintained at 37°C and humidity above 70% and allowed to equilibrate for 10–15 min. An air-tight pre-heated glass syringe was used to repeatedly collect 40 ml (representing one puff) of ECIG smoke and injected it into the desiccator. Ten puffs were injected to mimic one vaping session. The inlet tube contained multiple sidewise apertures for an even spread of the ECIG smoke within the desiccator. The macrophages cultured at ALI were exposed to ECIG smoke or filtered air for 15 min, where after they were transferred to a cell incubator (37°C, 60% humidity and 5% CO₂) for 1 hour (h) until next exposure session. Macrophages were exposed total 3 times and 10 puffs in each exposure with 1 h between each exposure. Following completion of 3 exposures, the macrophages were incubated for various time points depending on the readout indicated below.

Cell viability and apoptosis assay

According to manufacturer instruction LDH assay (Thermofisher, Sweden) and annexin A5 assay (BD Bioscience, USA) were performed for cell viability and apoptosis respectively. Shortly, MQ was exposed to ECIG with or without nicotine and incubated for 18 hours. After the incubation, cell culture supernatant was used for LDH assay and cells were stained with annexin A5 and apoptosis was determined by flow cytometry.

ROS measurement

After the 3rd exposure to ECIG smoke, macrophages were incubated for 2 hours. After the incubation, basal media was removed, and both apical and basal side of the insert was washed three times with PBS. Five µM of Cell ROX reagent (Thermofisher, Sweden) in RPMI media was added both at the basal (500µl) and apical side (500µl) of the insert. After 30 minutes of incubation, cells were washed 3 times
with PBS, trypsinized, resuspended in PBS and collected into flow cytometry tubes. In presence of reactive oxygen species (ROS), CellROX reagent generate fluorescence which is proportional to ROS level. The total ROS level was measured by flow cytometry, and median fluorescent intensity (MFI) was presented as level of ROS generation.

**Malondialdehyde measurement**

Macrophages were incubated for 18 hours after the 3rd exposure. According to the manufacturer instruction malondialdehyde (MDA) was measured from the cell lysate using MDA assay kit (Sigma Aldrich, Sweden). MDA-modified protein was investigated by FITC-labeled MDA antibodies (antibodies against MDA-modified protein, Abcam UK) using flow cytometry.

**Phospholipid measurement**

According to manufacturer protocol, levels of phospholipids in ECIG liquid and ECIG vapor condensate were measured by phospholipid assay kit (Abcam, UK).

**Macrophage phenotypic markers**

After the 3rd exposure to ECIG smoke, the macrophages were incubated for 18 hours. After the incubation, cells were trypsinized and stained with antibodies that are M1 markers (PerCp5.5-labeled CD86 and PE-labeled CD11C) and M2 marker (FITC-CD206 (BD bioscience, US). The expression of these cell surface markers were investigated by flow cytometry, and the expression level was presented as median fluorescence intensity.

**RT-qPCR**

After the 3rd exposure to ECIG smoke, the macrophages were incubated for 6 hours. After the incubation period, mRNA was extracted by RNA extraction mini kit (Qiagen, Germany). cDNA was synthesized from 300ng of RNA by cDNA synthesis kit (Applied biosystem, Germany) and 100 ng of cDNA was used for RT-qPCR. PCR amplification primers were selected according to earlier study (14). Housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The expression level of *glutathione peroxidase (GPx)*, *glutathione peroxidase (GPx4)*, *IL-6*, *TNF-α*, *IL-12*, *IL-1β* and *IL-10* was calculated by delta-delta CT methods.

**ELISA**

After the 3rd exposure, cell culture media was collected after 18 hours and secreted cytokines including IL-6, TNF-α, IL1-β, IL-8, IL-12A and IL-10 levels were measured by ELISA duoset (Biotechne, UK). In addition, cell lysates were prepared after 6 hours of 3rd exposures and HSP60 was measured from the cell lysates using ELISA duoset (Biotechne, UK).

**CD36 expression**
Surface expression of lipid scavenger receptor CD36 (BD Bioscience, US) was measured by flow cytometry after 18 hours of ECIG exposure and expression levels were presented as median fluorescent intensity.

**Lipid accumulation assay**

Lipid accumulation was measured by flow cytometry and microscopy. After ECIG exposure, according to manufacturer protocol, cells were stained with lipid staining reagent BODIPY (Sigma Aldrich, Sweden) and lipid accumulation was investigated by flow cytometry and confocal microscopy. Median fluorescent intensity was presented as the level of expression from Flow cytometry measurement and microscopic image was presented after developed by image J software.

**CD36 silencing**

According to manufactural protocol, CD36 was silenced with ShRNA (Santa cruz, Germany). In brief, differentiated macrophages were cultured with serum free medium in 6 wells plate and transfected with CD36 shRNA or with a control shRNA. After 6 hours of transfection, 10% FBS was added to the culture medium. After 72 hours, more than 70% reduced expression of CD36 was confirmed at gene and protein level by qRT-PCR and flow cytometry, respectively.

**TLR4 but not TLR2 expression**

MQ were incubated for 18 hours after the 3rd exposure of ECIG. After the incubation, MQ were trypsinized and stained with TLR2 and TLR4 antibodies (BD Bioscience, US) and the surface expression of TLR2 and TLR4 was investigated by flow cytometry and the expression level was presented as median fluorescence intensity.

**Phagocytosis**

Phagocytosis of *E. coli* particles (FITC labeled) by MQ as investigated according to manufacturer protocol (Vybrant™ Phagocytosis Assay Kit, Thermo Fisher, Sweden). In short, MQ culture at ALI were exposed to ECIG smoke as described above. After overnight incubation, apical side of the insert was washed with RPMI media and *E. coli* particles in 100µl of media was added on the top of the MQ. After 3 hours of incubation, MQ was trypsinized, transferred into 96 wells plates and the plates were read at 485 excitation wavelengths by microplate reader (Bioteknik, US). Percentage of phagocytosis was calculated and compared between control and exposed condition. As described above, MQ were exposed to ECIG and incubated for 18 hours after the 3rd exposure and stained with CD35 (complement receptor1) and CD64 (Fc receptor) antibodies (BD bioscience, US) to detect surface expression. The expression of these cell surface markers was analyzed by flow cytometry, and the expression level was presented as median fluorescence intensity (MFI).

**Statistical analysis**
Each experiment was performed with MQ from 3 donors (N = 3), and technical replicates n = 2–3 from each donor. The results were expressed as median and interquartile ranges (25th–75th percentiles) followed by non-parametric statistical analysis. Within each group, the comparisons between control and ECIG exposure with or without nicotine were performed by Friedman test and followed by Wilcoxon signed-rank test. In all tests, difference with a P value \( \leq 0.05 \) was as considered significant. Statistical significance in comparison to clean filtered air (sham) to all treatment condition expressed with * and between exposure with or without nicotine was expressed with #. All the data were analyzed using the GraphPad Prism 8.30 software.

**Results**

**Cell viability and apoptosis**

ECIG with or without nicotine did not affect viability of the MQ (data not shown). ECIG did not induced apoptosis neither with nor without nicotine. Although, there was a trend of minimal induction of apoptosis in response to ECIG with nicotine, but statistically not significant (Supp. Fig:1).

**ECIG induced oxidative stress**

Oxidative stress was assessed by measuring the level of ROS production. The level of ROS generation increased significantly in response to ECIG smoke exposure both with or without nicotine (Fig:1-A). The level of oxidative stress related genes \( GPx, GPx4 \) and \( HMOX1 \) (Fig:1B-D) were significantly increased in response to ECIG vapor both with and without nicotine but \( GPx4 \) increased significantly more in presence of nicotine (Fig: 1C). In addition, stress related protein HSP60 was significantly increased in response to ECIG smoke exposure both with and without nicotine, and significantly more in presence of nicotine (Fig:1-E).

**ECIG smoke induced lipid accumulation and lipid peroxidation**

ECIG liquid and ECIG vapor condensate both contained phospholipids (Supp.Fig:2) Next lipid peroxidation was measured in ECIG smoke exposed MQ. Both ECIG smoke with and without nicotine, but primarily with nicotine induced the lipid peroxidation product MDA in MQ (Fig:2-A). Exposure to ECIG both with and without nicotine, but significantly more with nicotine induced MDA-modified protein (Fig:2-B). Furthermore, lipid scavenger receptor expression (CD36) was increased in response to ECIG smoke both with and without nicotine, but primarily without nicotine (Fig:2-C). In addition to lipid scavenger receptor, lipid accumulation was significantly increased in ECIG (both with or without nicotine) smoke-exposed MQ (Fig:2, D-E).

**ECIG smoke induced pro-inflammatory macrophage polarization**

ECIG exposure resulted in increased expression of pro-inflammatory M1 macrophage polarization markers CD86 and CD11C. M1 markers were significantly induced both in presence and absence of nicotine compared to sham, where CD86 marker showed significantly higher expression in absence of
nicotine compared to nicotine containing ECIG vapor (Fig:3-A). However, ECIG exposure did not significantly induce M2 macrophage marker CD206 (Supp. Fig:3). The expression of pro-inflammatory cytokines IL-8 and IL-1 beta were significantly increased at gene level whereas the level of TNF, IL-6, IL-12A and IL-10 were not significantly affected at gene level after exposure to ECIG smoke (Fig:3-B). In addition, exposure to ECIG significantly increased the release of the inflammatory cytokines IL-6, IL-8 and IL-1 beta both in presence and absence of nicotine (Fig: 3-C). However, the release of the anti-inflammatory cytokine IL-10 was significantly reduced both in presence and absence of nicotine compared to sham (Fig: 3-C)

**CD36 silencing inhibited lipid accumulation and macrophage polarization**

Inhibition of CD36 expression (more than 70% inhibition) by CD36 ShRNA suppressed ECIG smoke-induced lipid accumulation in MQ (Fig:4-A). In addition, ECIG smoke-induced expression of macrophage polarization markers CD86 and CD11C were inhibited significantly by CD36 silencing (Fig:4, B-C).

**ECIG exposure induced TLR4 but not TLR2 expression**

In comparison to air (sham) exposure, ECIG exposure with or without nicotine did not affect the expression of TLR2 but induced the expression of TLR4 on MQ (Fig: 5)

**ECIG exposure induced decrease in phagocytosis of E.coli**

ECIG smoke exposure both with and without nicotine, affected phagocytosis activity of MQ. In comparison to filtered air (sham), in ECIG smoke-exposed MQ a reduced uptake of *E.coli* particles was seen (Fig:6-A). CD36 silencing recovered phagocytosis activity in ECIG-smoke exposed MQ (Fig:6-A). Furthermore, after ECIG smoke exposure, a reduced expression of phagocytosis receptors CD35 and CD64 was noticed both after exposure to ECIG with and without nicotine, but significantly more with nicotine regarding CD35 expression (Fig:6-B).

**Discussion**

Current study investigated possible mechanisms of harmful effects of vaporized E-cigarette products (ECIG smoke) in macrophage (MQ) cultured at ALI. Our data highlight the importance of MQ in lung lipid homeostasis and overall function of the innate immunity against ECIG. Severe harmful effect of ECIG exposure has been reported recently in clinical cases, but mechanisms of the harmful effects are still not elucidated. Different factors including *in vitro*, and *in vivo* experimental set-up, cell culture condition, biological difference, exposure or inhalation difference between animal and human makes it difficult to translate experimental data to real life. Here in the current study, we investigated MQ phenotype and function in connection with lipid homeostasis after exposure to electronic cigarette smoke vapor with or without nicotine in an *in vitro* experimental set-up, using peripheral blood monocyte derived MQ cultured at ALI. To study function of MQ in airways, this experimental set-up is more realistic in comparison to traditional submerged cultures including cell lines. To our knowledge, this is the first study where
Monocyte-derived macrophages were successfully cultured at ALI condition and exposed to ECIG smoke. Redox homeostasis especially in response to reactive oxygen species (ROS) by antioxidants play an important role in cellular function including signaling and metabolic activities. Here, ECIG smoke exposure with or without nicotine induced ROS generation in MQ. In response to higher ROS, antioxidants such as expression levels of GPx and GPx4 were increased. Transport or augmentation of GPx is an important step to balance redox homeostasis\(^\text{17}\). As expected, in response to ECIG-induced oxidative stress, the level of GPx was upregulated at gene level. GPx4 is more specific to lipid peroxidation-induced ROS generation. ECIG with or without nicotine, but primarily with nicotine induced GPx4 at gene level. The finding indicates that ECIG exposure in presence of nicotine increased cellular lipid peroxidation. In addition, HMOX1 is also induced in response to oxidative stress. While the function is broad, HMOX1 play a role in inflammation and macrophage polarization. Anti-inflammatory function of HMOX1 is suggested to be protective against the injury induced by hypoxia\(^\text{18}\). A randomized clinical trial suggested an ECIG- induce hypoxia\(^\text{19}\). However, here we identified induction of HMOX1 after exposure to ECIG smoke both with and without nicotine, which is in line with the earlier study which indicated ECIG induce hypoxia in the airway. Cellular protective function against oxidative damage is a complex phenomenon where HSP may play an important role. Among all HSP, HSP60 is more conserved in prokaryotic and eukaryotic cells and HSP60 is involved also in inflammation\(^\text{20}\). HSP60 production was induced after ECIG smoke exposure both with and without nicotine, but primarily with nicotine. Induction of HSP60 stimulated the proinflammatory effect including increased expression of proinflammatory cytokines\(^\text{20}\), as well as activation of immune cell has been reported\(^\text{20}\). Although we did not rule out whether ECIG-induced excessive HSP60 trigger the inflammatory response, but ECIG-induced HSP60 upregulation may have potential role to induce inflammatory condition in the airways. Since GPx4 induction indicates lipid peroxidation, we measured the lipid peroxidation product malondialdehyde (MDA). Our result showed ECIG exposure with or without nicotine, but primarily with nicotine induced the lipid peroxidation product MDA, which is consistent with the GPX4 induction. Earlier studies\(^\text{21, 22, 23}\) suggested vitamin E acetate (VEA) of ECIG as potential causative factor for oxidative damage-induced lung injury. Recently, one study showed that VEA exposure increased the level of MDA in a murine model\(^\text{23}\), which was suggested as a pathway for systemic inflammation in murine model of EVALI. However, the study claimed that VEA can be converted to vitamin E by alveolar macrophages. When vitamin E is a lipid soluble antioxidant, the connection between vitamin E and lipid of ECIG exposure is warranted for further investigation. Against pollutants and microorganisms, alveolar macrophages form the first line of defense and use a variety of pattern recognition and scavenger receptors to sense and phagocytose pathogens \(^\text{24}\). MQ is a known phagocytic cell type, but dynamic factors in MQ including macrophage phenotype, difference in maturation tissue migration have been identified. All these factors play important role in macrophage function\(^\text{24}\). However, we investigated whether lipid accumulation in macrophages differ in presence or absence of nicotine. Even though, the lipid peroxidation differed, but lipid accumulation was similar both with and without nicotine.

It is expected that in presence or absence of nicotine the lipid accumulation is identical in MQ since ECIG smoke exposure did not affect cellular lipid synthesis rather uptake from ECIG lipid. However, lipid
regulation has a potential effect on macrophage polarization and reprogramming (25). Surface expression of CD86 is a M1 specific marker, in addition, CD11C is also a M1 type of macrophages polarization marker. ECIG induced both CD86 and CD11C expression after ECIG exposure both with and without nicotine. We determined that ECIG-induced macrophage polarization is not nicotine-dependent, but future studies are warranted to test different flavors of ECIG and the effect of nicotine without flavors to investigate the role of nicotine in ECIG-induced effect. Our finding suggested that ECIG-induced macrophage polarization is not dependent on lipid peroxidation. However, CD206, a M2 specific marker was also investigated, but the expression of CD206 was not consistent in all donors after ECIG exposure. M2 specific macrophages play a role in the inflammatory resolution process, which is most important in the repair process. While in response to stimuli or infection pro-inflammatory function is activated, activation of anti-inflammatory function is a common phenomenon to balance this inflammation. However, in response to some stimuli, cellular anti-inflammatory function can be impaired. Our findings suggest that exposure to ECIG may impair the repair activity and/or anti-inflammatory function of macrophages. We measured M1 and M2 specific pro and anti-inflammatory cytokines and chemokine both at gene and secreted protein level. IL-8 and IL-1 beta was upregulated both at secreted protein and gene level. In addition, IL-6 was upregulated in secreted protein level. Cytokine secretion is regulated at many levels; at the level of transcription and translation and post-translationally at the endoplasmic reticulum, Golgi complex, and at/or near the cell surface(26). Although, following ECIG exposure the M2 MQ specific surface marker CD206 expression was not affected significantly, but M2 specific cytokine IL-10 was decreased significantly, suggests ECIG exposure may not affect polarization towards M2 MQ, but the function of M2 MQ.

Earlier clinical cases and animal data indicated ECIG-induced alteration of lipid homeostasis with increased lipid accumulation in macrophages, which is in line with our finding that showed ECIG-induced altered lipid homeostasis that regulated that inflammatory reaction. There are not sufficient analytical data on the content of phospholipid in ECIG liquids. We identified existence of lipid specifically phospholipid in ECIG liquid. Interestingly, in vapor condensate with or without nicotine we detected decreased level of lipids, compared to the pure ECIG liquids with or without nicotine (Supp.Fig:2). This might be explained that heat during puffing affect structure of phospholipids in the ECIG liquid. Phospholipid has context dependent effects, where phospholipids in intact or truncated form induced different cellular responses (27). In addition, oxidized phospholipids induced pro-inflammatory effects have been reported in several studies(28) (29, 30). Future studies to elucidate whether the lipids undergo structural changes after puffing and by this cause inflammation should be considered. Among all phospholipids, phosphatidylcholine is the most abundant phospholipid in cells. Phosphatidylcholine’s structure consist of arachidonic acid, glycerol palmitic acid and phosphocholine. Phospholipid induced functional alteration in airway is not well studied. A recent finding (31) showed that oxidized phospholipid induced oxidative stress and loss of viability in primary human epithelial cells BEAS-2B and Calu-3 cell lines, as well as epithelial cell barrier dysfunction, suggesting that phospholipids directly might contribute to disease development. However, peroxidation of arachidonic acid in phospholipids generate MDA. Lipid peroxidation-induced pro-inflammatory effect is now well established in healthy and diseased
conditions including respiratory inflammation (32). The lipid peroxidation product MDA and its potential role of inducing inflammation is still under investigation, and role MDA-modified protein has been implicated in disease(33). Our earlier studies showed that MDA modified human serum albumin changed peptide sequences and thus induced inflammation(33). In this current study, we identified that ECIG exposure induced MDA-modified cellular proteins. In the earlier study, we showed that uptake of MDA-modified serum albumin by inducing polarization of M1 macrophages(33). In this study CD36 silencing reduced lipid accumulation and thus inhibited M1 macrophage polarization. The MDA are potentially produced from ECIG-lipid and modify intracellular protein which may triggered M1 polarization. Future studies are recommended to identify the MDA-modified specific proteins which may be responsible for the M1 phenotype alteration. Since macrophage phenotypes decide the function of macrophages, we next investigated whether phagocytic activity of macrophages was affected by exposure to ECIG. The phagocytosis of *E.coli* were reduced after ECIG exposure whereas CD36 silenced MQ recovered the phagocytosis activity of macrophages significantly. Lipid laden macrophages have reduced phagocytic activity(30, 34). Our findings indicated that lipid uptake or disrupted lipid homeostasis were causative factors of reduced phagocytosis of *E.coli* by altering macrophage phenotype. FC gamma receptor (CD64) and complement receptor (CD35) are phagocytic receptors(35–37), CD35 mediated phagocytosis is complement dependent whereas CD64 is antibody dependent(38, 39). Although our cell culture condition was antibody free, we identified that both CD35 and CD64 were downregulated in response to ECIG exposure, primarily with nicotine, which indicated that ECIG-induced lipid accumulation may affect those receptors and thus reduce uptake of *E.coli*. Investigation to clarify whether inhibition of lipid uptake affect CD35 and CD64 expression will be of further interest. TLR including TLR2 and TLR4 play important role in innate immunity including pathogen recognition. In the macrophage mediated immune response including phagocytosis of bacteria, TLR2 and TLR4 signaling is a hallmark in defense reaction and bacterial phagocytosis by macrophages is promoted by TLR ligands (40). In our experiment TLR2 were not affected by ECIG exposure but TLR4 expression increased after ECIG exposure. TLR4 are key receptors which are induced by both infectious and non-infectious agents and triggers pro-inflammatory response. Oxidized phospholipids suggested as an inhibitor of TLR signaling(41) which might potentially play a role in reduced phagocytosis. Moreover, reduced phagocytic activity induced by exposure to ECIG, can potentially increase the risk of infection. Incidence of pneumococcal pneumonia and pneumococcal nasopharyngeal colonization are increased in cigarette smokers(42, 43). Further investigations are required to understand ECIG effect in bacterial pneumonia.

To our knowledge this is the first study on human monocyte-derived macrophages cultured at air liquid interface to investigate health effects of ECIG exposures. ECIG alter our lung lipid homeostasis and thus induced inflammation by activating M1 type macrophages and impaired their phagocytic function. These effect could potentially cause lung inflammation and exacerbation in diseases condition. Further investigation is needed to study cellular mechanisms in the presence of airway epithelial cells and to clarify in addition to lipid uptake whether cholesterol and phospholipid synthesis pathways are affected by ECIG exposure.
Declarations

Consent for publication

We give our consent to the publisher for the publication the content of the whole manuscript.

Author Contributions

MR conceived and designed the study, performed experiments, and wrote the manuscript. SS performed experiments and statistical analysis, contributed to study design and wrote manuscript. MI performed experiment and contributed in manuscript preparation. SU and KG conceived the study, contributed to study design and manuscript writing. LP conceived the study, contributed to study design, manuscript writing and supervised the entire study. All the authors read and approved the manuscript.

Ethics approval and consent to participate

NA.

Competing interests

All the authors declare that they have no competing interests.

Availability of the data and materials

Not applicable

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Supplementary Material

Supplementary materials were included separately.

Data Availability Statement

NA

References


Figures
Figure 1

ECIG smoke exposure induced oxidative stress in presence or absence of nicotine. Reactive oxygen species production was induced in response to ECIG exposure (A). Anti-oxidant (GPx) and lipid peroxidation specific antioxidant (GPx4) was upregulated by ECIG exposures with or without nicotine but GPx was increased primarily in presence of nicotine (B) and (C). HMOX1 in gene level was increased in response ECIG exposure (D). HSP60 expression was increased in response to ECIG exposure both with nicotine and without nicotine but primarily with nicotine (E). Statistical significance (p≤0.05) between Sham and exposure (nicotine or without nicotine) condition was indicated as *. Statistical significance between nicotine and without nicotine was indicated as #.
Figure 2

ECIG exposure induced lipid peroxidation and lipid accumulation. ECIG smoke exposure induced lipid peroxidation product malondialdehyde (MDA) in macrophages (A) and MDA-modified proteins (B). Lipid scavenger receptors CD36 (C) and lipid accumulation were increased in macrophages (D) and (E). Statistical significance ($p \leq 0.05$) between Sham and exposure (nicotine or without nicotine) condition was indicated as *. Statistical significance between nicotine and without nicotine was indicated as #.
Figure 3

Effects of ECIG smoke exposure on macrophage polarization and inflammatory response in macrophages cultured at air-liquid interface. ECIG vapor induced macrophage polarization markers CD86 and CD11c (A). The exposure induced change in level of inflammatory cytokines at gene level (B) and inflammatory cytokines in secreted protein level (C). Statistical significance (p≤0.05) between Sham and exposure (nicotine or without nicotine) condition was indicated as * and between nicotine and without nicotine was indicated as #.
CD36 silencing inhibit lipid accumulation and macrophage polarization. Lipid uptake was inhibited in CD36 silenced macrophages (A). M1 type macrophage polarization markers CD86 (B) and CD11C (C) expression was reduced in CD36 silenced macrophages. ECIG (A) Statistical significance ($p \leq 0.05$) between Sham and exposure (nicotine or without nicotine) condition was indicated as * in each group (Control group, CD36 shRNA group and control shRNA group). CD35 and CD64 expression were downregulated in ECIG-exposed macrophages.
Figure 5

ECIG exposure induced TLR4 expression. ECIG-exposed MQ induced surface expression of TLR4 by presence or absence of nicotine in ECIG but primarily in presence of nicotine whereas TLR2 expression was not affected significantly. Statistical significance (p \leq 0.05) between Sham and exposure (nicotine or without nicotine) condition was indicated as *. Statistical significance between nicotine and without nicotine was indicated as #.
Figure 6

ECIG inhibit phagocytosis of *E.coli*. Macrophages reduced phagocytosis of *E. coli* after exposed to ECIG (A) Statistical significance ($p \leq 0.05$) between Sham and exposure (nicotine or without nicotine) condition was indicated as * in each group Control group, CD36 shRNA group and control shRNA group). CD35 and CD64 expression were downregulated in ECIG-exposed macrophages (B) and (C). Statistical significance ($p \leq 0.05$) between Sham and exposure (nicotine or without nicotine) condition was indicated as *.

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